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AWARD NUMBER DAMD17-97-1-7134

TITLE: Role of mp 170 Seprase in Breast Cancer

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REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Annual (20 Jun 97 - 19 Jun 98)

4. TITLE AND SUBTITLE
Role of mp 170 Seprase in Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7134

6. AUTHOR(S)
Quang Nguyen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University Medical Center
Washington, DC 20007

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Seprase, a cell surface serine type gelatinase with M_r 170 kDa whose expression is associated with melanoma invasiveness, is composed of two identical subunits of M_r 97 kDa. Recent evidence indicated that the Seprase subunit is identical to Fibroblast Activation Protein α (FAPα). To characterize and define the role of this molecule in cancer, human Seprase/FAPα cDNA was cloned and stable transfected in two human epithelial carcinoma cell lines SW-13 and MCF-7. Unexpectedly, overexpression of Seprase/FAPα has no apparent effect on the proliferation, matrix adhesion and matrigel invasion of these cells. Preliminary site-directed mutagenesis studies suggested that the region coding for the signal/anchorage domain of the molecule and this intact domain are probably essential for Seprase/FAPα mRNA stability and the dimerization of the subunits, respectively. Ribozyme constructs targeted Seprase/FAPα mRNA have been made. However, despite the *in vitro* cleavage activity, these ribozyme constructs are not effective in abrogating the endogenous Seprase/FAPα level in highly metastatic melanoma 1205LU cells.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
30

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Thuy Nguyen 7/14/98

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INTRODUCTION

Degradation of basement membranes and stromal extracellular matrix is an essential step in tumor invasion and metastasis. The process is mediated by a wide spectrum of induced proteases derived from both neoplastic and tumor stromal cells. Some of the implicated proteases include: the matrix metalloproteases, serine plasminogen activators, cysteine cathepsins B and L, and aspartic cathepsin D (1,2).

mp170 Seprase is originally identified in the laboratory of Dr. Wen-Tien Chen at Georgetown University as a 170 kDa membrane-associated gelatinase that is expressed in highly aggressive melanoma LOX cells (3). The enzyme is a homodimer of 97 kDa subunits which in the monomeric form do not possess gelatinolytic activity (4). Furthermore, it has been reported that under certain conditions, Seprase can be mobilized to specialized ventral surface membrane structures called "invadopodia" of invasive tumor cells (5). Surprisingly, a proteinase complex of M_r 170 kDa displaying strong gelatinase activity is also found in the nonionic detergent extract of non-invasive human embryonic lung WI-38 fibroblasts. This proteinase complex is not only upshifted to a high M_r > 200 kDa complex in gelatin substrate zymograms by monoclonal antibody (mAb) D8 specific for Seprase but also by mAb F19 directed against Fibroblast Activation Protein α, FAPα (Fig. 1A). Under denaturing conditions, immunoprecipitates of the radiolabeled extract using either mAb D8 or mAb F19 conjugated agarose beads reveal a predominant protein component of M_r 95 kDa which apparently lacks gelatinolytic activity (Fig. 1B). Thus, it appears that the proteinase complex is a dimer of FAPα molecules, and the gelatinolytic activity is solely associated with the dimeric form. Moreover, the fibroblast proteinase complex and its gelatinolytic activity are abolished upon exposure to pH < 6.0 or temperature > 65°C, whereas high salt concentrations and reducing agents have no apparent effects (Fig. 2). Inhibitor studies also indicate that the fibroblast proteinase complex is a serine-type gelatinase since its gelatinolytic activity is completely abolished by AEBSF [4-(2-aminoethyl-benzenesulfonyl fluoride hydrochloride], but not by aprotinin, benzamidine, E-64, phenanthroline, EDTA or pepstatin A. The biochemical properties of the fibroblast proteinase complex are very similar to those recently described for Seprase isolated from LOX cells (6). Thus, it appears that the mp170 Seprase subunit is identical to FAPα.

Fibroblast activation protein α is originally described by Dr. Wolfgang J. Rettig as a cell surface glycoprotein with M_r 95 kDa, and it is recognized by mAb F19 in cultured fibroblasts (7). Immunohistochemical studies have shown that FAPα is selectively induced *in vivo* in stromal fibroblasts of human breast, ovarian, colorectal, bladder and lung carcinomas, and activated fibroblasts of healing wounds; whereas it is generally absent in normal tissues (8). FAPα is widely expressed *in vitro* in cultured normal fibroblasts, but its expression is generally absent or downregulated in transformed mesenchymal cell lines (9). A full length cDNA for human WI-38 fibroblast FAPα was isolated by expression cloning in COS-1 cells (10). Analysis of the deduced amino acid sequence reveals that FAPα is a type II integral membrane protein consisting of a large extracellular domain, a transmembrane segment and a short cytoplasmic tail. In the extracellular matrix domain, the molecule contains a conserved catalytic triad as that of a related serine protease dipeptidyl peptidase IV (DPPIV). However, the enzymatic activity displayed by FAPα molecules has not been demonstrated by Dr. Rettig's group. Recently, the FAPα gene has been mapped to human chromosome 1q21-23 by fluorescence *in situ* hybridization (11).

Due to a restricted *in vivo* expression pattern of FAPα, ^{131}I-mAb F19 directed against FAPα has been utilized in phase I clinical work for imaging carcinoma lesions (12). It appears that FAPα is a potentially useful target for diagnosis and therapy of epithelial cancers. However, little is known about the biological function of FAPα or mp170 Seprase (Seprase/FAPα). Therefore, the main goal of this research project is to characterize Seprase/FAPα and to examine its role in breast cancer. This annual report presents the work performed during the first year of the Army Grant DAMD 17-97-7134.

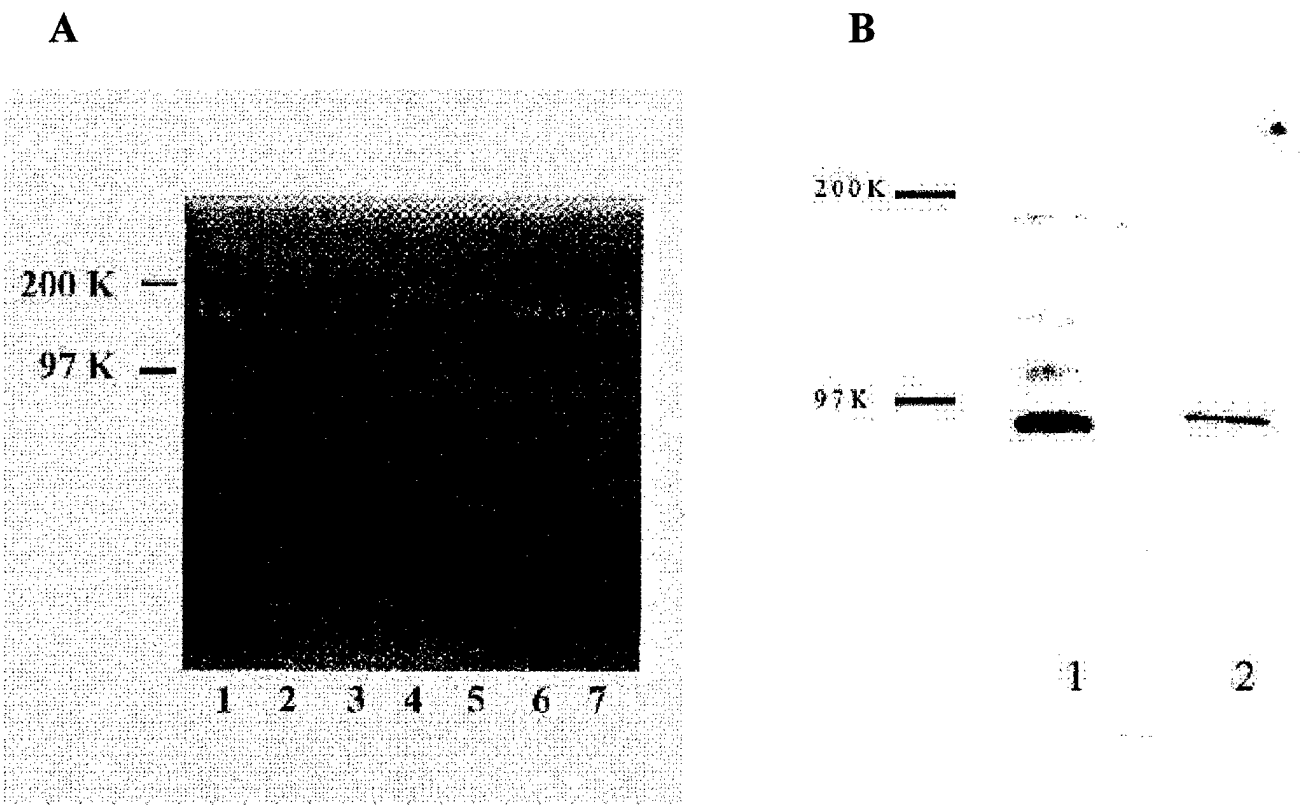


Fig. 1. FAPα in dimeric form possesses gelatinolytic activity. A. Aliquots of partially purified membrane extracts of WI-38 fibroblasts were preincubated without (lane 1), or with 5ul and 10 ul of hybridoma containing either mAb F19 specific for FAPα (lanes 2,3), or mAb F4 directed against dipeptidyl peptidase IV (lanes 4,5), or mAb C37 directed against a p90 plasma membrane glycoprotein (lanes 6,7) at room temperature for 4 h prior to gelatin zymogram analysis. B. Partially purified ^{35}S-labeled membrane extracts of WI-38 fibroblasts that were eluted from either mAb F19-agarose beads (lane 1) or mAb D8 (specific for Seprase)-agarose beads (lane 2) under boiling conditions were analyzed by SDS/PAGE, followed by autoradiography.

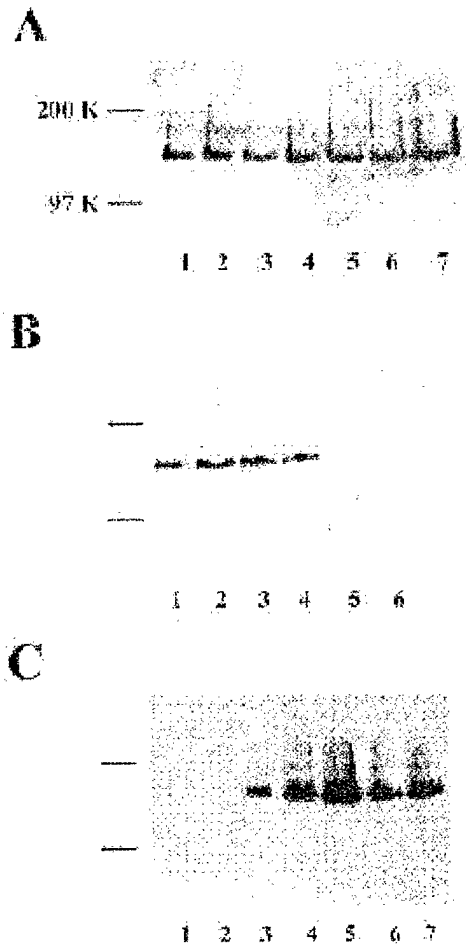


Fig. 2. Effect of Salt, Temperature and pH on the stability of FAPα dimers. Partially purifed WI-38 fibroblast membrane extracts were treated under various NaCl concentrations: 0.15, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0 M (Panel A), or various temperatures: 25, 37, 55, 65, 75, 85°C (Panel B), or various pHs: 4, 5, 6, 7, 8, 9, 10 (Panel C) prior to immunoblotting analysis using mAb F19.

BODY

I. Molecular cloning of Seprase/FAPα.

On the basis of similar biochemical properties exhibited by the WI-38 fibroblast gelatinase complex to those described for mp170 Seprase extracted from LOX cells, and the apparent cross-reactivity between mAb F19 and mAb D8, it appears that the genes encoding for Seprase subunit and FAPα are identical. In order to pursue structure/function studies of this molecule, its full lenth cDNA was constructed by the reverse transcription-polymerase chain reaction (RT-PCR) approach using FAPα oligonucleotide primers.

Methods.

RT-PCR and plasmid construction. Total cellular RNA was isolated from WI-38 fibroblasts using the RNA STAT-60 kit (Tel-Test "B", Inc., Friendswood, TX). Two overlapping cDNA fragments covering the entire open reading frame of Seprase/FAPα were prepared by RT-PCR. Reverse transcription was carried out at 37 °C for 60 min in 20 uL of reverse transcription buffer containing 5 ug total RNA, 0.5 mM deoxynucleotide triphosphate mixture, 40 units RNAsin (Promega Biotec, Madison, WI), 0.5 ug oligo $(dT)_{12\text{-}18}$ primer, 10 mM dithiothreitol and 200 units Moloney murine leukemia virus reverse transcriptase. After denaturation at 94 °C, the reaction mixture was adjusted to a final volume of 100 ul of 1x PCR buffer containing 5 units of Taq DNA polymerase and 1 ug each of appropriate FAPα oligonucleotide primers. For FAPα cDNA fragment covering nucleotides 171-1094 (GenBank accession no. U09278, submitted on April 29, 1994), the following primers were used: 5'-AAGACAGAATTAGCTAAC-3' and 5'-GCCAACTGAAATAATAATCACTTG-3'. For FAPα cDNA fragment covering nucleotides 818-2485, a 6x histidine tag and stop codons in all three reading frames, these primers were used: 5'-GAAATGCTTGCTACAAAATATG-3' and 5'-TCAGCTAATTAGTGATGGTGATGGTGATGGTCTGACAAAGAG-3'. PCR was performed using 30 cyles of 45 s at 94 °C, 30 s at 55 °C and 1 min 30 s at 72 °C. PCR products were subcloned into the pCR3.1 mammalian expression vector using the eukaryotic TA cloning kit (Invitrogen, San Diego, CA). A full length FAPα cDNA in the expression vector was constructed by the ligation of the *BamH1-Cla1* fragment of the former clone, with the *Cla1-Xho* fragment of the latter clone, and the *BamH1-Xho* pCR3.1 vector.

Plasmid sequencing. cDNA was sequenced using the PRISM ready reaction dye deoxy terminator cycle sequencing kit (Pekin Elmer, Norwalk, CT) on the ABI Model 373A DNA sequencer (Lombardi Cancer Center Macromolecular Synthesis and Sequencing Core Facility).

Results & Discussions.

A representative full length cDNA sequence coding for Seprase/FAPα isolated from a clone numbered 10, pSF10, is shown in Fig. 3. The sequence is essentially similar to that reported by Scanlan *et al.* (10). However, there are some notable differences. Most interestingly, there are three additional guanine nucleotides at different sites in the sequence between nucleotides 2081-2220,

1 aagacagaattagctaactttcaaaaacatctggaaaa***atg***aagacttgggtaaaaatcg
61 tatttggagt tgccacctct gctgtgcttg ccttattggt gatgtgcatt gtcttacgcc
121 cttcaagagttcataactct gaagaaaata caatgagagcactcacactgaaggatattt
181 taaatggaac attttcttat aaaacatttt ttccaaactg gatttcagga caagaatatc
241 ttcatcaatc tgcagataac aatatagtac tttataatat tgaaacagga caatcatata
301 ccatttttgag taatagaacc atgaaaagtgtgaatgcttc aaattacggc ttatcacctg
361 atcggcaatt tgtatatcta gaaagtgatt attcaaagct ttggagatac tcttacacag
421 caacatatta catctatgac cttagcaatg gagaatttgt aagaggaaatgagcttcctc
481 gtccaattca gtatttatgc tggtcgcctg ttgggagtaa attagcatat gtctatcaaa
541 acaatatcta tttgaaacaa agaccaggag atccacctttttcaaataaca tttaatggaa
601 gagaaaataaaatatttaat ggaatcccag actgggtttatgaagaggaa atgcttgcta
661 caaaatatgc tctccggtgg tctcctaatg gaaaattttt ggcatatgcg gaatttaatg
721 atacggatat accagttatt gcctattcct attatggcga tgaacaatat cctagaacaa
781 taaatattcc atacccaaag gctggagcta agaatcccgt tgttcggata tttattatcg
841 ataccacttaccctgcgtat gtaggtccccaggaagtgcctgttccagca atgatagcct
901 caagtgatta ttatttcagt tggctcacgt gggttactga tgaacgagta tgtttgcagt
961 ggctaaaaagagtccagaatgtttcggtcc tgtctatatg tgacttcagg gaagactggc
1021 agacatgggattgtccaaagacccaggagcatatagaagaaagcagaactggatgggctg
1081 gtggattctt tgtttcaaga ccagttttca gctatgatgc catttcgtac tacaaaatat
1141 ttagtgacaaggatggctac aaacatattc actatgtcaa agacactgtggaaaatgcta
1201 ttcaaattacaagtggcaag tgggaggcca taaatatattcagagtaacacaggattcac
1261 tgttttattc tagcaatgaa tttgaagaat accctggaag aagaaacatc tacagaatta
1321 gcattggaagctatcctcca agcaagaaat gtgttacttgccatctaaggaaagaaaggt
1381 gccaatattacacagcaagt ttcagcgact acgccaagta ctatgcactt gtctgctacg
1441 gcccaggcatccccatttcc acccttcatg atggacgcactgatcaagaa attaaaatcc
1501 tggaagaaaacaaggaattggaaaatgctt tgaaaaatatccagctgcctaaagaggaaa
1561 ttaagaaact tgaagtagat gaaattactt tatggtacaa gatgattctt cctcctcaat
1621 ttgacagatc aaagaagtat cccttgctaa ttcaagtgta tggtggtccctgcagtcaga
1681 gtgtaaggtc tgtatttgct gttaattgga tatcttatct tgcaagtaag gaagggatgg
1741 tcattgcctt ggtggatggt cgaggaacag ctttccaaggtgacaaactcctctatgcag
1801 tgtatcgaaa gctgggtgtt tatgaagttg aagaccagat tacagctgtcagaaaattca
1861 tagaaatggg tttcattgat gaaaaaagaatagccatatg gggctggtcctatggaggat
1921 acgtttcatc actggccctt gcatctggaa ctggtctttt caaatgtggt atagcagtgg
1981 ctccagtctc cagctgggaa tattacgcgt ctgtctacac agagagattcatgggtctcc
2041 caacaaaggacgataatctt gagcactata agaattcaactgtgatggca agagcagaat
2101 atttcagaaa tgtagactat cttctcatcc acggaacggc agatgataat gtgcactttc
2161 aaaactcagcacagattgct aaagctctggttaatgcaca agtggatttc caggcaatgt
2221 ggtactctgaccagaaccacggcttatccg gcctgtccacgaaccacttatacacccaca
2281 tgacccactt cctaaagcag tgtttctctt tgtcagac ***catcaccatcaccatcactaat***
2341 ***tagctga***

Fig. 3. Nucleotide sequence of human WI-38 Seprase/FAPα in pSF10. Nucleotide substitutions and insertions of 3 extra guanidine nucleotides in the underlined sequence relative to the original FAPα cDNA sequence (10) are indicated in bold letters with shade. The start codon is shown in underlined bold italicized letters. Nucleotides coding for a 6x histidine tag and stop codons in all three reading frames are in bold italicized letters, selected ribozyme target sites are double underlined.

resulting in a change in the deduced amino acid sequence of 45 residues immediately following the putative active site serine residue. This frameshift renders the catalytic domain of FAPα to be more homologous to that of the related DPPIV. A revised sequence of FAPα submitted to GenBank on March 14, 1997 confirmed the presence of these extra guanine nucleotides that are apparently missed in the original sequence. Relative to the revised sequence of FAPα, our sequence also contains several nucleotide substitutions denoted by bold letters in shade in Fig.3, including: $C_{827}\rightarrow G_{827}$, $T_{845}\rightarrow C_{845}$, $A_{894}\rightarrow C_{894}$, $A_{1346}\rightarrow G_{1346}$, $G_{1519}\rightarrow A_{1519}$, $T_{2221}\rightarrow C_{2221}$ and $A_{2308}\rightarrow G_{2308}$. The indicated nucleotide positions are in reference to the revised GenBank FAPα cDNA sequence. The first four subsitutions result in the replacement of proline, tryptophan, lysine, isoleucine to alanine, arginine, threonine and valine, respectively. The other nucleotide substitutions are silent mutations. Recently, a very similar LOX Seprase cDNA sequence that was cloned by RT-PCR using FAPα primers was also reported (13).

II. Expression of Seprase/FAPα in cell lines.

The expression of Seprase/FAPα in various cell lines was next examined by Northern and Western blot analysis.

Methods.

Cell lines. The following cell lines were used in the study: Human embryonic lung WI-38 fibroblasts, mouse NIH-3T3 fibroblasts, Chinese hamster ovary cells, human umbilical cord endothelial cells HUV-EC-C, human adrenal adenocarcinoma SW-13, pleiotrophin transfected SW-13 (W28), human choriocarcinoma JEG-3 and JAR, human melanoma 1205LU, human breast cancer MCF-7, BT-474 and ZR75-1. Human melanoma 1205LU cell line was obtained from Dr. M. Heerlyn, Wistar Institute, Philadelphia. Other cell lines and the F19 mouse hybridoma producing mAb F19 specific for FAPα were purchased from the American Type Culture Collection (Rockville, MD).

Northern blots. Northern blot analysis was carried out as described previously (14). Total RNA (20 ug/sample) was fractionated on a 1% agarose gel containing 1.1% formaldehyde and 0.25 ug/ml ethidium bromide. The RNA was transferred onto a nylon transfer membrane (Micron Separations Inc., Westboro, MA) by capillary blotting overnight. The membrane was prehybridized at 42 °C for 16 h in 5x SSC, 50% formamide, 1x Denhardt's solution, 1% SDS and 100 ug/ml of denatured salmon sperm DNA. Hybridization was carried out in the prehybridization solution with heat-denatured α-^{32}P-labeled probe at 42 °C for 16 h. The membrane was washed in 2x SSC at room temperature for 5 min, followed by 2x SSC containing 1% SDS at 55 °C for 30 min, and 0.1x SSC at room temperature for another 30 min. After drying, the membrane was exposed to Kodak X-AR film at -70 °C with an intensifying screen. The *Hind III* fragment of pSF10 containing a Seprase/FAPα sequence between nucleotides 170 and 570 was used as a probe, and was purified using the QIAEX II gel extraction kit (Qiagen Inc., Valencia, CA). The cDNA probe was radiolabeled with [α-^{32}P]dCTP using the Rediprime random primer labelling kit (Amersham Life Science Inc., Arlington Heights, IL). The level of RNA sample loading was monitored by the intensity of ethidium bromide stained ribosomal RNAs.

Western blots. For Western blot analysis, cells were extracted in Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 1% Triton X-100 with 1 mM each of phenylmethylsulfonyl fluoride (PMSF) and ethylenediamine tetraacetic acid (EDTA). The cell lysate was extracted by slow stirring at 4 °C for overnight, and insoluble particulates were removed by centriguation at 10,000 x g for 15 min at 4 °C. The extracted proteins were resolved on 7.5% SDS/polyacrylamide gels under reducing conditions, followed by electroblotting onto nitrocellulose membranes (15). The membranes were probed with mouse mAb F19, followed by alkaline phosphatase conjugated rabbit anti-mouse IgG under conditions described previously (16). Immunoreactive proteins were then visualized by the electrochemiluminescence detection system.

Protein determination. To load samples with similar protein contents for gel analysis, total protein in cell extracts was estimated using the Bio-Rad protein assay (Hercules, CA). Briefly, 2-5 uL of cell extracts were diluted to 0.8 mL with water, to which 0.2 mL of concentrated Bio-Rad protein reagent was added. After 10 min at room temperature, total protein in cell extract samples was estimated by measuring absorbance at 590 nm. Bovine serum albumin was used as a standard.

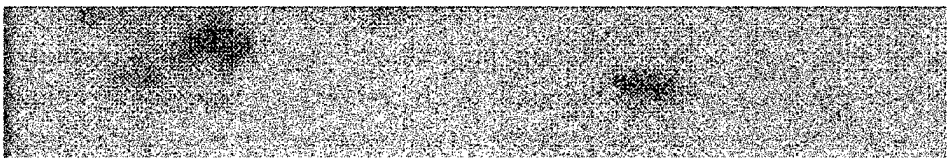
Results & Discussions.

Among the cell lines tested, Seprase/FAPα mRNA was detected in human embryonic lung WI-38 fibroblasts and human melanoma 1205LU (Fig. 4A, lanes 2 and 9 respectively). This was confirmed by the presence of the dimeric form of the protein in their cell extracts as shown by the Western blot probed with mAb F19 (Fig. 4B, lanes 1 and 4). It is surprising that no Seprase/FAPα expression was detected in mouse NIH-3T3 fibroblasts even though FAPα appears to be a characteristic *in vitro* induced marker for cultured fibroblasts (9). Similar to previous findings (7,9), there is an apparent lack of Seprase/FAPα expression in most tumor cell lines analyzed here. The absence of Seprase/FAPα expression in the human adrenal carcinoma cell line SW-13 is useful because this cell line is easily transfectable and it supports a high level of gene expression from the CMV (cytomegalovirus) promoter present in the mammalian cell expression vector pCR3.1 (17,18). Thus, it is chosen to be transfected with Seprase/FAPα constructs for structure/function studies of the molecule. Due to a relatively high level of Seprase/FAPα expression, the highly invasive melanoma cell line 1205LU appears suitable to be transfected with ribozyme constructs targeted Seprase/FAPα in order to examine the effect of the molecule on the invasive properties of this cell line.

III. Characterization of human adrenal carcinoma SW-13 and breast tumor MCF-7 cells overexpressing Seprase/FAPα.

To examine the role of Seprase/FAPα on tumor growth and invasiveness, full length Seprase/FAPα cDNAs were transfected into human adrenal carcinoma SW-13 and non-invasive breast tumor MCF-7 cells. As shown in Fig. 4A, both parental cell lines do not express Seprase/FAPα mRNA. Stable transfectants were then analyzed for their proliferative and invasive properties.

A.



1 2 3 4 5 6 7 8 9 10 11 12

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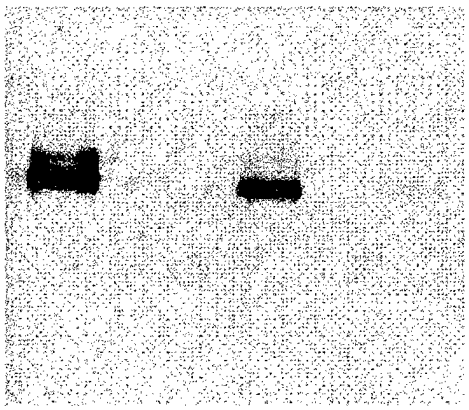


1 2 3 4 5 6

Fig. 4. Expression of Seprase/FAPα in cultured cell lines. A. Northern blot analysis. Lane 1, NIH-3T3; lane 2, WI-38; lane 3, CHO; lane 4, HUV-EC-C; lane 5, SW-13; lane 6, W28; lane 7, JEG-3; lane 8, JAR; lane 9, 1205LU; lane 10, MCF-7; lane 11, BT-474; lane 12, ZR75-1. For each cell type, 20 ug total RNA was analyzed as described in Methods, with the *Hind III* cDNA fragment of pSF10 plasmid used as a probe. The arrow indicates the position of Seprase/FAPα mRNA. B. Western blot analysis. Lane 1, WI-38; lane 2, CHO; lane 3, NIH-3T3; lane 4, 1205LU; lane 5, SW-13; lane 6, JEG-3. The cell lysates were subjected to immunoblotting analysis using mAb F19.

Methods.

Transfections. Cells were transfected with either empty vectors or pSF10 vectors containing full length Seprase/FAPα cDNA using LipofectAMINE (Life Technologies, Gaithersburg, MD). Cells (2×10^5) at approximately 50% confluency were incubated with 1 ug plasmid DNA and 7 uL of LipofectAMINE reagent in Opti-Mem I reduced serum medium for 5 h at 37 °C in 5% CO_2 atmosphere. The transfection mixture was then replaced with a complete growth medium (IMEM with 10% FCS). After 48 h post-transfection, cells were cultured in a complete growth medium containing G418 (500 ug/mL), and stable transfected cells were selected in the presence of this drug for another 3 weeks. Clonal cell lines were obtained by limited dilution of a suspension of mass transfected cells using 96 well plates, and individual clones were expanded in the presence of G418.

Gelatin zymography. The gelatinolytic activity of expressed Seprase/FAP dimers in transfected cells was analyzed by SDS/PAGE under non-reducing and non-boiling conditions on 7.5% gels containing 1 mg gelatin/mL (19). Following electrophoresis, the gels were washed with 2.5% Triton X-100 solution for 30 min, then incubated in Tris-buffered saline containing 1mM EDTA for 24-48 h at 37 °C. After staining gels with Coomassie Brilliant Blue R-250 and destaining, the gelatinolytic activity was observed as a clear band on a blue background. To concentrate the level of extracted Seprase/FAPα in the detergent phase for analysis, cells were lysed in 50 mM Tris, 150 mM NaCl, pH 7.5 buffer containing 1% Triton X-114 (20).

Cell proliferation assays. A colorimetric assay using cell proliferation reagent WST-1 was used to measure cell proliferation and viability (Boehringer Mannheim). Briefly, cells were cultured in 96 well plates (500-2,000 cells in 100 uL IMEM medium with 10% FCS/well) at 37 °C in 5% CO_2 atmosphere for various incubation periods (1 to 6 days). After each incubation period, 10 uL of the cell proliferation reagent WST-1 was added to each well, and the cells were incubated at 37 °C for 1-3 h until optimal absorbance at 450 nm could be read using a Molecular Devices microplate reader. A reference wavelength of 650 nm was used.

Cell adhesion assays. Cell adhesion assays were performed as described by Brooks *et al.* (21). Briefly, 96-well plates were incubated with 50 ug/mL fibronectin (Life Technologies) or 50 ug/mL collagen (CelTrix, Santa Clara, CA) or 2% porcine skin gelatin (Sigma, St. Louis, MO) in sterile phosphate buffered solution (PBS) for 16 h at 4 °C. Plates were washed three times with PBS, then incubated with 3% BSA for at least 1h at room temperature to avoid nonspecific cell attachment. Cells suspended in serum free media (50,000 cells/well) were allowed to attach at 37 °C for 15 min. Plates were rinsed with PBS twice to remove unattached cells, and attached cells were stained with 0.5% crystal violet at room temperature for 15 min. Cell-associated crystal violet was eluted with 0.1 M sodium citrate/50% ethanol solution, and plates were read at 600 nm.

Soft agar assays. Anchorage-independent growth was assessed by soft agar assays under conditions described by Wellstein *et al.* (19). Briefly, a single cell suspension (10,000-20,000 cells in 0.36% agar in the presence of various concentrations of basic fibroblast growth factor (bFGF) was layered onto a bottom layer containing 0.6% agar in a 35 mm-tissue culture dish. Dishes were then incubated at 37 °C in a 5% CO_2 atmosphere for 10-14 days. The number of colonies >60 um in diameter formed after the incubation was counted using an Omnicon 3800 Tumor colony analyser (Imaging Products International, Chantilly, VA).

Chemoinvasive assay. Cells (1×10^5) suspended in serum-free IMEM medium containing

0.1% BSA were added to the upper chamber of Biocoat Matrigel Invasion Chamber (Becton Dickinson Labware, Bedford, MA) containing an 8 um pore-size PET (polyethylene teraphthalate) membrane coated with a uniform layer of Matrigel basement membrane matrix. IMEM medium containing 10% FCS and 0.1% BSA which was used as a source of chemoattractant was placed in the lower compartment of the chamber. After 24-48 h of incubation at 37 °C, the filters were fixed, stained with Diff-Quik as suggested by the manufacturer. Cells on the upper surface of the filter were wiped away with a cotton swab. Cells on the lower surface of the filter were observed and counted under a phase contrast microscope. The chemotaxis assays were performed in a similar fashion after an overnight incubation, using uncoated filters with 10% FCS as a source of chemoattractant.

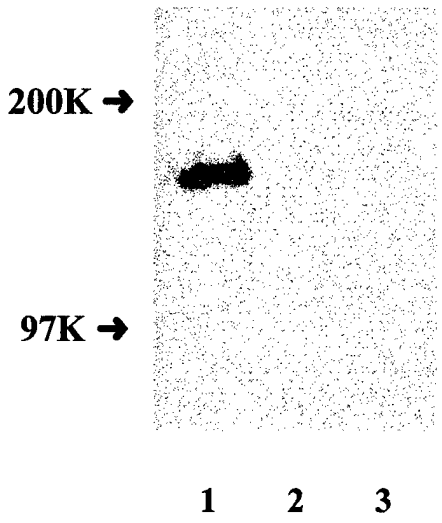
Results & Discussions.

Stable SW-13 and MCF-7 mass transfectants expressing Seprase/FAPα were isolated and characterized to examine the effect of the molecule on the properties of these epithelial cancer cell lines. Western blot analysis revealed a strong mAb F19-reactive band of M_r 170 kDa present in the cell extract derived from pSF10 transfected SW-13 cells, but not in those of mock transfected or parental cells (Fig. 5A). To ascertain that expressed Seprase/FAPα dimers still possess gelatinolytic activity, aliquots of aqueous and detergent phase cell extracts in Triton X-114 buffer were subjected to gelatin zymography. As expected, a lysis band was detected in the detergent phase cell extract of pSF10 transfectants, but not in that of mock transfectants (Fig. 5B). The lysis band due to Seprase/FAPα dimers present in the detergent phase cell extract of WI-38 fibroblasts was used as a positive control. There was no gelatin lysis band detected in aqueous fractions of all cell extracts. Similar results were obtained for cell extracts derived from mock and pSF10 transfected MCF-7 cells. Thus, stable SW-13 and MCF-7 mass transfectants express Seprase/FAPα dimers recognized by mAb F19, and the dimers possess gelatinolytic activity.

1. Cellular proliferation. Upon characterization of these stable mass transfectants, we found that Seprase/FAPα has no apparent effect on their proliferation in plastic cultures as shown in Fig. 6. An isolated SW-13 clonal cell line expressing high level of Seprase/FAPα exhibited a similar proliferation pattern as those obtained for mock and pSF10 mass transfectants. Similarly, both mock and pSF10 transfected MCF-7 cells displayed similar growth curves. In anchorage indedendent growth soft agar assays, Seprase/FAPα does not promote colony formation in these cells. In the presence of bFGF at various concentrations, no significant difference in the number of colony formed was found between Seprase/FAPα expressing and non-Seprase/FAPα expressing cell populations. Fig. 7A shows representative soft agar assay results of SW-13 mock transfectants and high expressing Seprase/FAPα clonal cells at two different tested bFGF concentrations (0.1 and 0.5 ng/mL). In this assay, 0.5 ng/mL is the optimal bFGF to yield the maximum number of colony formed by SW-13 cells. It is apparent that Seprase/FAPα has no significant effect on the proliferative properties of two epithelial cancer cell lines SW-13 and MCF-7 tested here.

2. Matrix adhesion. The effect of Seprase/FAPα on the adhesion of SW-13 and MCF-7 transfectants on fibronectin, collagen and gelatin was also examined. Under the conditions used, SW-13 cells adhere rapidly to fibronectin-coated wells within 15 minutes of cell plating, whereas little cell attachment is observed in wells coated with either collagen or gelatin or control uncoated wells. The expression of Seprase/FAPα in SW-13 cells does not alter the adhesive properties of this cell line on

A.



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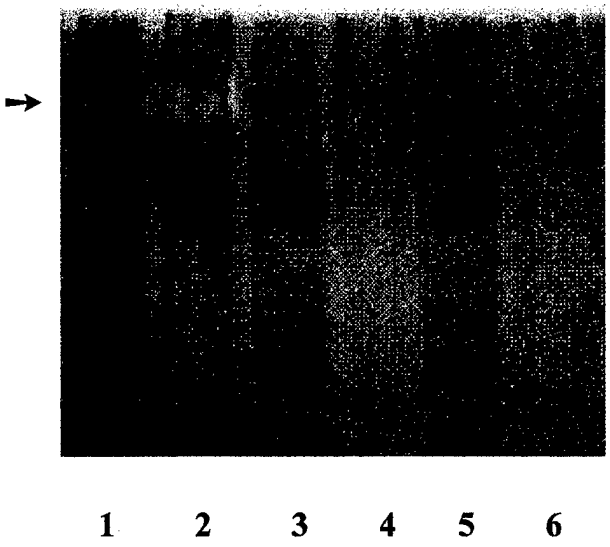
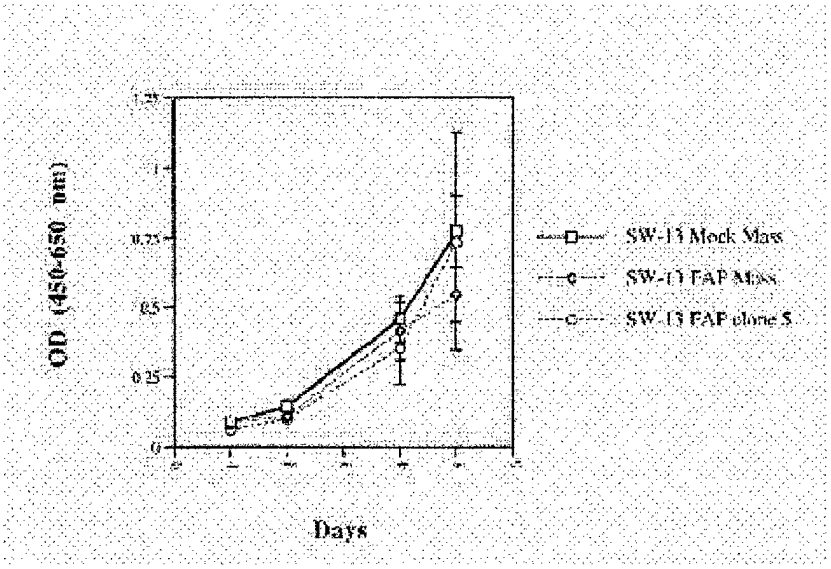


Fig. 5. Overexpression of Seprase/FAPα in human adrenal carcinoma SW-13 cells. A. Cell extracts derived from pSF10 transfected (lane 1), mock transfected (lane 2) and parental SW-13 cells (lane 3) were subjected to immunoblotting analysis using mAb F19. B. Aqueous (lanes 1,3, 5) and detergent (lanes 2, 4, 6) aliquots of Triton X-114 cell extracts derived from WI-38 fibroblasts (lanes 1,2), mock transfected (lanes 3,4) and pSF10 transfected SW-13 cells, respectively, were analyzed by gelatin zymography. An arrow indicates the position of the hydrolysis band.

A.



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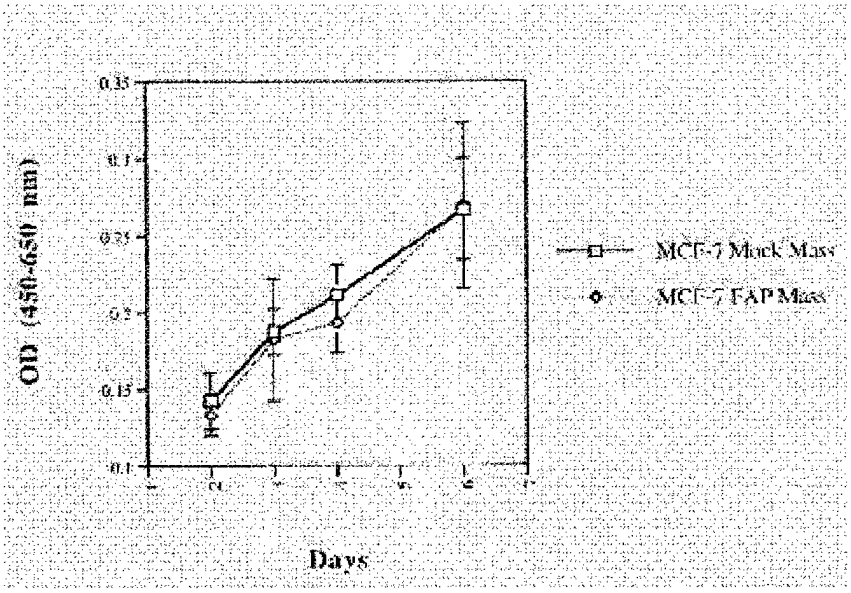
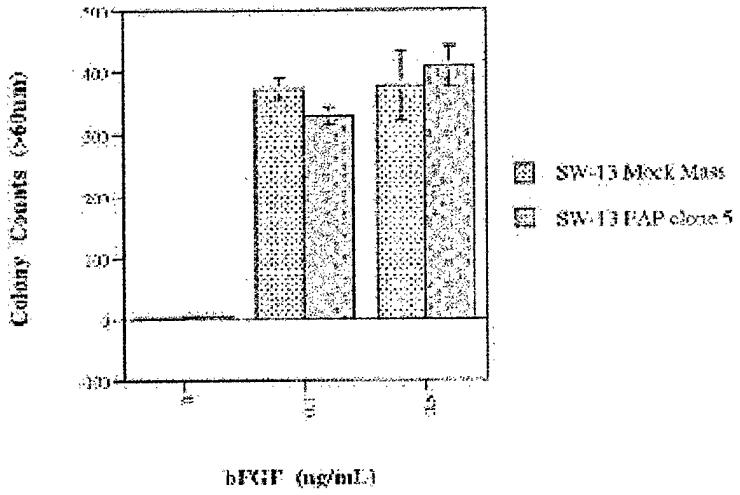


Fig. 6. Effect of Seprase/FAPα on the proliferation of SW-13 and MCF-7 cells. SW-13 (Panel A) and MCF-7 (Panel B) cells (500-2,000 cells/well) were cultured in 96 well plates in IMEM medium containing 10% fetal calf serum for various time intervals at 37°C. At each time interval, the tetrazolium salt WST-1 was added to each well, and cell proliferation was quantitated on the basis of the cleavage of the WST-1 substrate by mitochondrial dehydrogenases in viable cells. The absorbance wavelength for the product is 450 nm with the reference wavelength at 650 nm. Each point on the plot represents the mean value of a triplicate with the standard deviation.

A.



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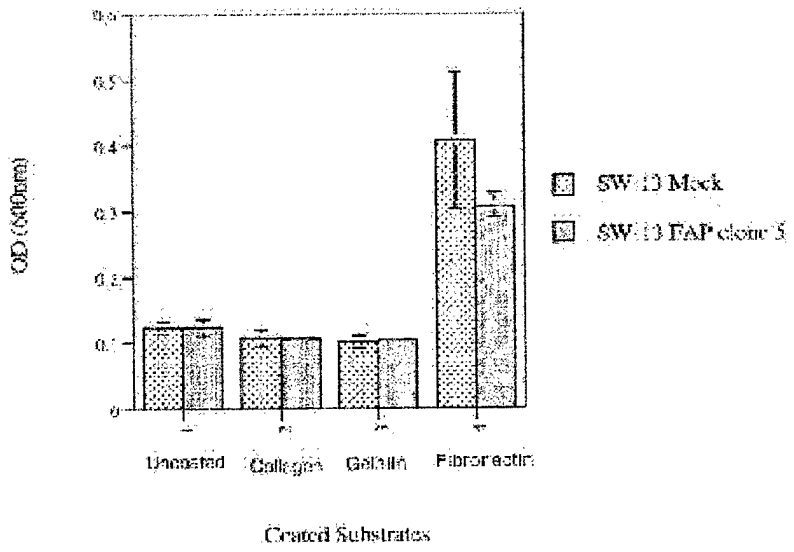


Fig. 7. Effect of Seprase/FAPα on SW-13 cells in soft agar and cell adhesion assays. Mock transfected mass and a clone expressing high level of Seprase/FAPα were assayed for anchorage independent growth in soft agar (Panel A) under various concentrations of basic fibroblast growth factor (0, 0.1, and 0.5 ng/mL), and for cell adhesion (Panel B) on collagen, gelatin and fibronectin substrates as described in Methods. Each bar represents the mean value of a triplicate with the standard deviation.

the tested substrates as shown in Fig. 7B. Similarly, the adhesion properties of MCF-7 cells on fibronectin, collagen and gelatin are not modulated by the expression of Seprase/FAPα. The data may also suggest that Seprase/FAPα does not have a high affinity for fibronectin, collagen or gelatin. In contrast, the highly related DDPIV is thought to function as a cell adhesion molecule through its interaction with collagen types I, III and possibly with fibronectin (22, 23), in addition to its function as an exopeptidase.

3. Matrigel Invasion. The Biocoat matrigel invasion chamber is used as an *in vitro* system to assess the effect of Seprase/FAPα on the invasive characteristics of both SW-13 and MCF-7 cells. Fig. 8A showed that very few mock and pSF10 transfected SW-13 cells (<5 cells per insert) are capable of traversing through the matrigel layer. In chemotaxis assays, although relatively higher cell counts (10-15 cells per insert) were obtained, there was no apparent difference in cell numbers migrating to the lower chamber in response to FCS between the mock and pSF10 transfected SW-13 cell populations (Fig. 8B). Murine embryonic 3T3 fibroblasts that have reasonable chemoinvasive and chemotactic responses in these assays were used as positive control cells (24). Similar observations were made when serum-free conditioned medium of NIH-3T3 fibroblasts was used as a chemoattractant source instead of FCS. It is clear that in this assay system, Seprase/FAPα expression has neither effect on the invasion nor the motility of SW-13 cells. Similar observations were made for mock and pSF10 transfected MCF-7 cells. The lack of matrigel invasion displayed by both Seprase/FAPα expressing SW-13 and MCF-7 cells is quite unexpected since Seprase/FAPα is thought to be a major protease mediating the dissolution of tumor cell extracellular matrix and its expression has been attributed to melanoma invasiveness (3, 4). The weak chemotactic response exhibited by these stable transfectants could be a partial reason, although there is no direct association between chemotactic and chemoinvasive responses (24).

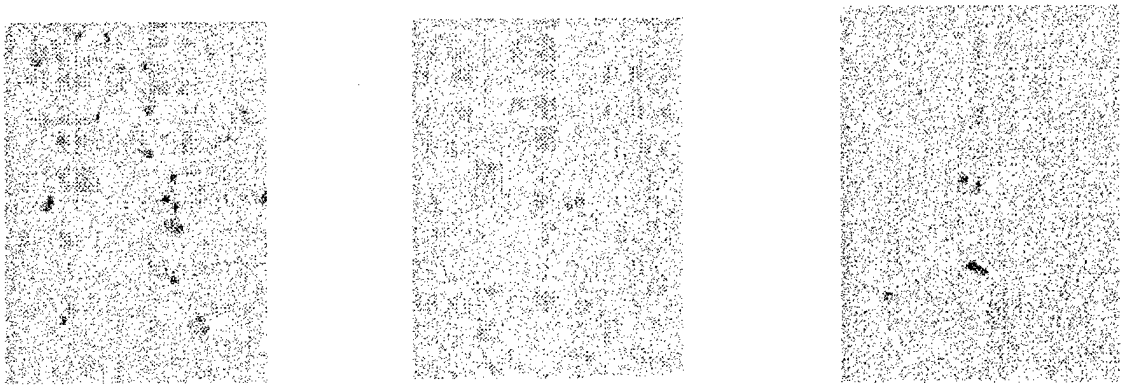
Thus far, Seprase/FAPα has no apparent effect on various cellular aspects (proliferation, adhesion, motility and invasion) in two human epithelial cancer cell lines, SW-13 and MCF-7. With respect to the unique *in vivo* expression pattern of Seprase/FAPα which is exclusively localized on activated fibroblasts of malignant cancers (8), *in vitro* model systems involving co-culture experiments between Seprase/FAPα expressing and Seprase/FAPα non-expressing mesenchymal cells with epithelial cancer cells may have yielded some useful information regarding to the functional role of this molecule in cancer. This is the direction that will be pursued for future work.

IV. Construction of secretable Seprase/FAPα molecules.

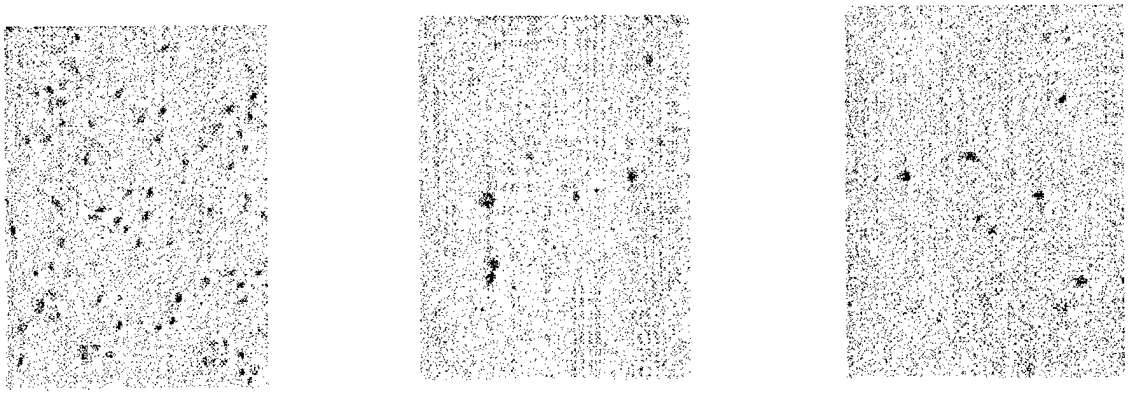
The biochemical characterization of Seprase/FAPα is essential in the understanding of its role in carcinoma. Determining the natural substrate specificity of the enzyme is an aspect that may yield invaluable insights. Thus far, gelatin is the only known substrate for Seprase/FAPα and its cleavage site is not known. Due to the limitation of obtaining sufficient purified membrane-bound Seprase/FAPα for biochemical characterization, the construction of secretable form of Seprase/FAPα molecules, particularly the soluble dimers that possess gelatinolytic activity, was proposed. Using the following strategies, Seprase/FAPα mutant cDNA constructs were made by site-directed mutagenesis.

1. Pertubation of the signal/anchor sequence. It has been shown that a partial truncation of

A. Matrigel



B. Uncoated



3T3 **SW-13 Mock** **SW-13 FAPα**

Fig. 8. Effect of Seprase/FAPα on the chemoinvasion and chemotaxis of SW-13 cells. Cells in serum-free media were added to the upper chamber of Biocoat Matrigel Invasion chambers containing either Matrigel coated (Panel A) or non-coated (Panel B) inserts. Fetal calf serum (10%) used as a chemoattractant was placed in the lower chamber. After incubation at 37 °C for 24-48 h (Panel A) or for 12 h (Panel B), migrating cells attached onto the lower surface of the inserts were stained with Diff-Quik. Mouse embryonic 3T3 fibroblasts were used as positive control cells.

the signal/anchor domain of DPPIV results in the secretion of this ectopeptidase (25). In comparison to other domains, there is a low homology in the signal/anchor region between Seprase/FAPα and DPPIV (10). Nevertheless, a Seprase/FAPα mutant cDNA that has a deletion in the sequence encoding amino acids 4-12, $p95_{\Delta 4\text{-}12}$, was constructed.

2. Insertion of the RNRQKR sequence immediately after the signal/anchor sequence. The RNRQKR sequence contains the consensus motif RXKR which is recognized and cleaved by the furin-like family of intracellular processing endoproteases. It has been shown that both stromelysin-3 and MT1-MMP (membrane type 1- matrix metalloproteinase) are constitutively processed at this cleavage site motif in mammalian cells (26). A Seprase/FAPα mutant, $p95_{RNRQK}$ that has the RNRQK sequence inserted between L_{26} - R_{27} was made.

3. Swapping the signal/anchor domain of Seprase/FAPα with the signal peptide domain of secreting stromelysin-3. The signal peptide of stromelysin-3 with the sequence MKSLPILLLLCVAVCSA was used to replace the signal/anchor sequence MKTWVKIVFGVATSAVLALLVMCIVLR of Seprase/FAPα. This mutant is designated as p95/SL-3spd .

Methods.

Site-directed mutagenesis. It was performed by the polymerase chain reaction overlap extension method (27). Basically, two cDNA fragments with overlapped ends were generated in two separate PCR reactions using wild type Seprase/FAPα cDNA in pCR3.1 vector as a template, with a specific pair of mutagenic oligonucleotides and a pair of flanking primers. The sequences of specific pairs of mutagenic oligonucleotides used to generate the above mutants are shown below.

1. $p95_{\Delta 4\text{-}12}$:

M K T_3 T_{13} S A V L A
5'-CATCTGGAAAAATGAAGACTACCTCTGCTGTGCTTGCC-3'
3'-GTAGACCTTTTTACTTCTGATGGAGACGACACGAACGG-5' (anti-sense)

2. $p95_{RNRQK}$:

R N R Q K R_{27} P S R V
5'-CGCAACCGACAGAAGCGCCCTTCAAGAGTTC-3'
3'-CTACACGTAACAGAATGCGTTGGCTGTCTTCGC-5' (anti-sense)
M C I V L_{26} **R N R Q K**

3. p95/SL-3spd:

L L L L C V C V C S A Y P S R V H N
5'-CTACTGTTGCTGTGCGTGGCAGTTTGCTCAGCCTATCCTTCAAGAGTTCATAAC-3'
3'-GTAGACCTTTTTACTTCTCAGAAGGTTAGGATGACAACGACACGC-5' (antisense)
M K S L P I L L L L C

For flanking primers, a sequence between nucleotides 331-349 in the pCR3.1 vector, and an antisense sequence covering Seprase/FAPα nucleotides 620-643 were used. Their respective sequences are : 5'-CTTATGGGACTTTCCTAC-3', 5'-CTCATTTCCTCTTACAAATTCTCC-3'. The two generated PCR fragments were annealed, and further amplified by PCR in the presence of the two flanking primers to generate p95 cDNA cassettes that contain the mutations. The *BamH1-Bsm1* of newly generated PCR fragments were ligated to the *BamH1-Bsm1* pCR3.1 vector containing the region that encodes the remainder of p95. The mutated p95 constructs were verified by sequencing analysis.

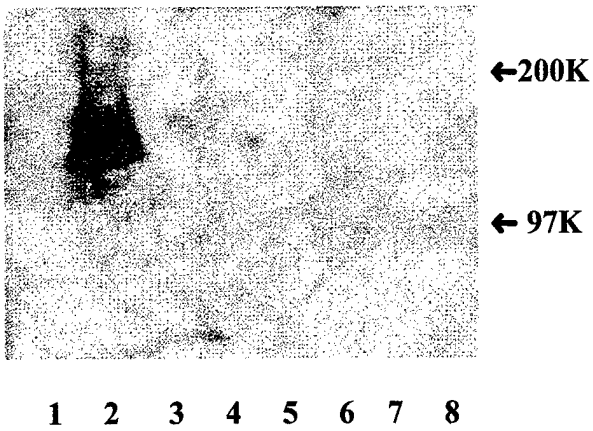
Cell transfection and analysis of mass transfectants. The mutated Seprase/FAPα constructs were transfected into SW-13 cells using the LipofectAMINE method under the same conditions as those used for the transfection of wild type pSF10 vector. After 3 weeks of G418 selection (0.5 mg/mL), stable mass transfectants of mutated Seprase/FAPα were obtained. Cells near confluency were cultured in serum-free or 10% FCS containing IMEM for at least 2 days. The culture media were collected, and cells were lysed in the extraction buffer containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100 with 1 mM each of EDTA and PMSF. Both the culture media and cell lysates were subjected to Western blot analysis using F19 mAb. In addition, total RNAs of the mutants were subjected to Northern blot analysis. A *HindIII* fragment of the pSF10 plasmid was used as a probe. To assess RNA sample loadings, a human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA control probe (CLONTECH, Palo Alto, CA) was used.

Results & Discussions.

Western blot analysis of stable mass trasfectants for $p95_{\Delta 4\text{-}12}$, $p95_{RNRQK}$ and p95/SL-3spd constructs revealed the absence of mAb F19 reactive band in their culture media and cell lysate extracts (Fig. 9A). To investigate whether the mRNAs of these mutant constructs were expressed, Nothern blot analysis was conducted. In comparison to the mRNA steady state level of wild type Seprase/FAPα derived from a stable transfecting mass, there is a drastic decrease in the message levels of all the mutants (Fig. 9B). The GAPDH levels are similar in all analyzed samples indicating that the lower message levels for the mutants are not due to lower RNA loadings. It may appear that the messages for the mutants are unstable. Although the mutant constructs were transfected into SW-13 cells under the same conditions as those used for the wild type construct, one can not completely rule out the observed differences in the steady state message levels are due to differences in transfection efficiency. The significant decrease in the message levels of the mutants may account for the absence of expected secreting Seprase/FAPα dimers recognized by mAb F19. However, pertubations in the signal/anchor domain of Seprase/FAPα could adversely affect the dimerization of the molecule, and this may contribute to the lack of mAb F19 reactive band on the Western blot. Thus, whether the region encoding the signal/anchor domain of Seprase/FAPα plays a role in determining the mRNA stability and the signal/anchor domain is essential for the dimerization of the subunits, this is the work that is currently investigated in more details.

Due to the unexpected results obtained for the mutant constructs, I am focusing on expressing wild type Seprase/FAPα using the baculovirus expressing system in order to obtain sufficient purified dimers for the biochemical characterization studies. In addition to the high level of heterologous

A.



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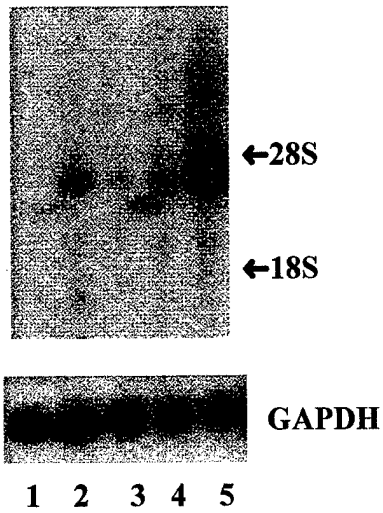


Fig. 9. Expression analysis of Seprase/FAPα mutants. A. Cells extracts (lanes 2,4,6,8) and serum-free cultured media (lanes 1,3,5,7) of SW-13 cells transfected with wild type pSF10 (lanes 1,2), $p95_{\Delta 4\text{-}12}$ (lanes 3,4), $p95_{RNRQK}$ (lanes 5,6), and p95/SL-3spd (lanes 7,8) constructs were subjected to immunoblotting analysis using mAb F19. B. Total RNA isolated from parental SW-13 cells (lane 1), and stable mass transfectants of $p95_{\Delta 4\text{-}12}$ (lane 2), $p95_{RNRQK}$ (lane 3), p95/SL-3spd (lane 4), and pSF10 (lane 5) constructs were subjected to Northern blot analysis. The *Hind III* cDNA fragment of pSF10 plasmid was used as a probe. A human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA control probe was also used to monitor equal RNA sample loadings.

gene expression of this system, the expression vector also contains a region encoding for a 6x His tag at the amino terminus of the expressed protein to simplify the purification process. Although, a SW-13 clone that expresses a relatively high level of Seprase/FAPα was obtained, the histidine tag that is introduced at the carboxy terminus of the protein (Fig.3) is apparently not exposed for binding to the nickel-coupled resin under non-denaturing conditions (Results not shown). Thus, difficulties in obtaining purified Seprase/FAPα dimers using cell extracts derived from this SW-13 clone were encountered.

V. Abrogation of Seprase/FAPα expression in tumor cells.

Concurrent to the Seprase/FAPα overexpression studies, attempts to investigate the biochemical effect of Seprase/FAPα downregulation in certain tumor cell lines using the ribozyme approach were made. In the initial study, three hammerhead ribozyme constructs targeting specifically Seprase/FAPα mRNA at different sites were constructed and transfected into a metastatic human melanoma 1205LU cell line. A hammerhead ribozyme consists of a catalytic center made up of a highly conserved sequence of 22 nucleotides and two arms that base-pair the ribozyme with target RNA sequences flanking the trinucleotide GUX, where X can be A, C or U. A ribozyme cleaves on the 3' side of the trinucleotide in the target RNA (28).

Methods.

Design of ribozyme constructs. Three potential ribozyme cleavage sites in the Seprase/FAPα mRNA which were selected are GUC_{703}, GUC_{852} and GUC_{2079}. A pair of sense and anti-sense oligocucleotides with complimentary 3' ends, covering the sequences of the catalytic center (underlined sequences) and flanking regions of the cleavage site are synthesized. Following are the sequences of respective pairs of oligonucleotides used to synthesize hammerhead ribozyme constructs targeting at the selected sites:

1. $Rbzfap_{703}$:

5'-GTTTTGATACTGATGAGTCCGTGAGG-3'
5'-AGCATATGTTTCGTCCTCACGGAC-3'

2. $Rbzfap_{892}$:

5'-ATTAGGACTGATGAGTCCGTGAGG-3'
5'-TCTGGTGGTTTCGTCCTCACGGAC-3'

3. $Rbzfap_{2079}$:

5'-TCCATAGCTGATGAGTCCGTGAGG-3'
5'-GGCTGGTTTCGTCCTCACGGA-3'

The ribozyme oligonucleotides were annealed, amplified by PCR under conditions previously described, then cloned into pCR3.1 vector using the TA-cloning method. The orientation and sequence of the ribozyme constructs were verified by sequencing analysis.

***In vitro* transcription reactions.** mRNA transcripts of the ribozyme constructs and their target substrates were synthesized *in vitro* using the T7 RNA polymerase promoter present in the pCR3.1 vector. A typical 20 uL transcription reaction contains 1 ug of linearized ribozyme or substrate containing plasmids with 1 mM each of ATP, CTP, GTP and UTP, 30 units T7 RNA polymerase, 40 units RNAsin in a transcription buffer of 40 mM Tris-HCl, pH 8.0, 6 mM $MgCl_2$, 10 mM dithioerythitol, 2 mM spermidine and 10 mM NaCl. The transcription reaction was incubated at 37 °C for at least 4 h. Prior to the termination of the transcription reaction by the addition of EDTA to a final concentration of 20 mM, 20 units of RNase-free DNase I were added to the reaction mixture and the reaction was incubated for another 20 min to remove the DNA template. The transcripts were precipitated with 2.5 M ammonium acetate and 2.5 volumes of ethanol. The pellet was washed twice with 70% ethanol, then air dried.

***In vitro* ribozyme cleavage reactions.** The ribozyme and target RNA transcripts were mixed in a 10 uL reaction mixture containing 50 mM Tris-HCl, pH 7.5 and 1 mM EDTA. The mixture was heated to 95 °C for 5 min, then incubated at 50 °C for 15 min. The cleavage reaction was started by the addition of $MgCl_2$ (5 mM - 10 mM), and the reaction mixture was incubated at 50 °C for 1 h. The reaction was stopped by the addition of 10 uL Stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% cyanocyanol). After heating the samples for 2 minutes at 70 °C, the cleavage products were separated on 6% polyacrylamide-7M urea gel in Tris-borate buffer (0.09M Tris-borate, 0.02M EDTA, pH 8.0) and visualized by silver staining as described by Palfner *et al.* (29). Basicly, the gel was washed once in 1% HNO_3 for 15 min, followed by 0.2% silver nitrate for 15 min. After a short wash with water, the gel was developed in a solution containing 3% Na_2CO_3, formaldehyde (0.05% of 37% stock), and sodium thiosulfate (2 mg/L). The gel was fixed with 10% acetic acid for 10 min.

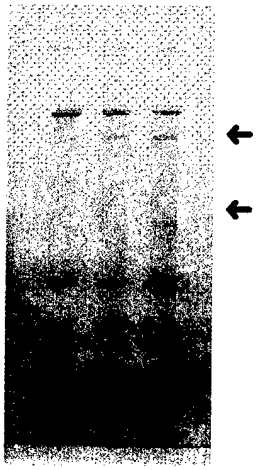
Ribozyme transfection. Stable mass and clonal transfected 1205LU cells with ribozyme constructs were isolated as described above. The concentration of G418 used to select transfected 1205LU cells is 1.5 mg/mL. The efficacy of the ribozyme constructs in abrogating the basal expression of Seprase/FAPα in 1205LU cells was assessed by both Western and Northern blots.

Results & Discussions.

Three hammerhead ribozyme constructs targeting Seprase/FAPα were made. A ribozyme mediated *in vitro* cleavage activity of a representative ribozyme construct $Rbzfap_{703}$ is shown in Fig.10A. $Rbzfap_{703}$ cleaved a segment of the Seprase/FAPα transcript containing the target site to yield two degraded transcripts of expected sizes (590 and 310 base pairs). The intensity of the degraded products increased at a higher concentration of $MgCl_2$. It is apparent that the ribozyme construct has *in vitro* cleavage activity and its activity appears to be specific.

All the ribozyme constructs were used in the transfection of 1205LU melanoma cells. Despite the demonstrated *in vitro* cleavage activity, none of the ribozyme constructs reduced significantly the basal level of Seprase/FAPα in stable mass transfectants. As shown in Fig.10B, the protein levels of Seprase/FAPα in stable mass transfectants of $Rbzfap_{703}$, $Rbzfap_{892}$ and $Rbzfap_{2079}$ are similar to

A.



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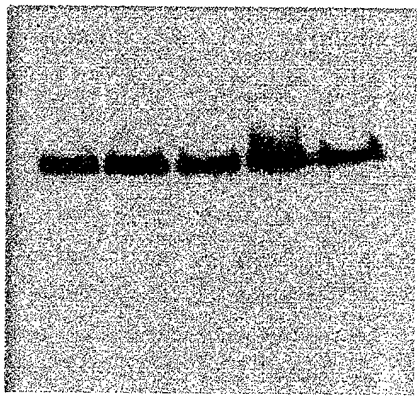
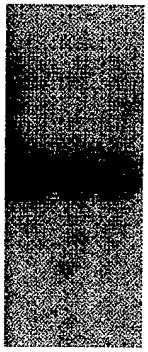


1 2 3 4 5

C.



1 2 3

Fig. 10. Ribozyme mediated cleavage of Seprase/FAPα. A. *In vitro* cleavage reactions: Reaction mixtures containing $Rbzfap_{703}$ and its substrate RNA transcripts in the absence (lane 1) or presence of 5 mM or 10 mM $MgCl_2$ (lanes 2 and 3, respectively) were incubated at 50 °C for 1 h. The cleavage products were separated and visualized on a silver stained 6% polyacrylamide-7M urea gel. Positions of the cleavage products were indicated by arrrows. B. Cell extracts of 1205LU mass transfected with $Rbzfap_{703}$ (lane 1), or $Rbzfap_{892}$ (lane 2), or $Rbzfap_{2079}$ (lane 3), or empty pCR3.1 vector (lane 4), and that of parental cell line (lane 5) were subjected to immunoblotting analysis using mAb F19. C. Nothern blot analysis of 1205LU mass transfected with empty pCR3.1 vector (lane 1), or $Rbzfap_{703}$ (lane 2), or $Rbzfap_{2079}$ (lane 3) using a *HindIII* cDNA fragment of pSF10 plasmid as a probe.

those of mock transfectants and parental cells. The protein data is further supported by similar Seprase/FAPα mRNA levels isolated from mock, $Rbzfap_{703}$ and $Rbzfap_{2079}$ transfectants (Fig. 10C). Clonal cell lines derived from $Rbzfap_{703}$ and $Rbzfap_{2079}$ mass transfectants were also isolated, however, none of the cell lines showed any subtantial reduction in the endogenous level of Seprase/FAPα.

It appears that the selected target sites on Seprase/FAPα are inaccessible *in vivo,* probably due to RNA folding and protein binding. It may be necessary to construct more ribozymes targeting at other sites of the molecule. The hammerhead ribozyme approach has been used successfully in suppressing the endogenous levels of various molecules including: a secreted growth factor pleiotrophin, a secreted basic Fibroblast growth factor-binding protein and transcriptional co-activators p300 and CBP (cAMP-response-element-binding protein (CREB)-binding protein) in various cell lines (30, 31, 32). In addition to 1205LU melanoma cells, the most effective ribozyme constructs will be transfected into WI-38 fibroblasts to investigate the effect of stromal Seprase/FAPα expression on the tumorigenicity and invasiveness of breast cancer cells in nude mice as proposed previously.

CONCLUSIONS

The dissolution of the basement membrane matrix by proteolytic enzymes is a critical step of the invasion/metastasis process of neoplastic cells. Among the various implicated proteases, mp170 Seprase' expression appears to be correlated to the invasiveness of melanoma cells (3,4). More recently, our biochemical data suggest that the genes encoding mp170 Seprase subunit and FAPα are identical, and that mp170 Seprase is a dimer of FAPα. The *in vivo* expression of FAPα is quite restricted and specificly induced in stromal fibroblasts of malignant epithelial carcinomas (8), and thus potentially be a contributing factor to the metastasis.

In the pursuit to characterize and define the role of Seprase/FAPα in breast cancer, a full length human embryonic WI-38 fibroblast FAPα cDNA was cloned by the RT-PCR approach. The isolated cDNA sequence is very similar to the revised GenBank FAPα cDNA, particularly with the presence of three extra guanine nucleotides in the region encoding for the catalytic active site of the molecule. These guanine nucleotides were apparently missed in the initial reported sequence (10). The isolated sequence is also similar to the LOX Seprase cDNA sequence (13). Transfection of this gene into human adrenal carcinoma SW-13 cells result in the presence of mAb F19 reactive molecules of M_r 170 kDa in the detergent phase of cell extracts, and they possess gelatinase activity as expected.

The effect of Seprase/FAPα on the proliferation and invasiveness of two human epithelial carcinoma cell lines SW-13 and MCF-7 was examined. Apparently, Seprase/FAPα has no effect on the proliferation of these cell lines in both anchorage dependent and anchorage-independent growth assays. Seprase/FAPα does not modify adhesion properties of these cell lines on fibronectin, collagen and gelatin substrates. Unexpectedly, overexpression of Seprase/FAPα does not enhance the invasive properties of SW-13 and MCF-7 cells. This result indicates that overexpression of Seprase/FAPα is not sufficient to promote the invasiveness of these epithelial carcinoma cells. However, it does not preclude any indirect role that Seprase/FAPα may participate in the invasion and metastasis of cancer cells.

In an effort to produce secretable Seprase/FAPα, pertubations of the signal/anchorage domain of the molecule including: a deletion of a segment between amino acids 3 and 13 ($p95_{\Delta 4\text{-}12}$), an insertion of a furin-cleavage site at the carboxy terminus of the domain ($p95_{RNRQK}$), and a swap with a signal domain of stromelysin-3 (p95/SL-3spd) were performed. Interestingly, comparing to the message level of the wild type, SW-13 transfected cells produce significantly lower message levels for all of these mutants. Furthermore, mAb F19 reactive molecules of M_r 170 kDa could not be detected in either culture media or extracts of cells transfected with the mutant constructs. This suggests that the region coding for the signal/anchorage domain of Seprase/FAPα is essential for the stabilization of its mRNA message and/or the signal/anchorage domain is critical for the subunit dimerization process.

Concurrently to the overexpression of Seprase/FAPα in non-invasive carcinoma cell lines, the abrogation of Seprase/FAPα expression in highly invasive melanoma 1205LU cells was performed using the ribozyme approach. Three ribozyme constructs cleaving on the 3' side of nucleotides numbered 703, 892, 2079 were made. Despite the *in vitro* cleavage activity, none of the ribozyme constructs made so far successfully decreases the endogenous Seprase/FAPα level of 1205LU cells.

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